

Polycyclic aromatic hydrocarbon-DNA adducts determined by semiquantitative immunohistochemistry in human esophageal biopsies taken in 1985

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Abstract

Esophageal endoscopic biopsy samples were obtained in 1985 in Linxian, China, a region with very high esophageal cancer incidence rates, and where ingested food is known to contain substantial amounts of polycyclic aromatic hydrocarbons (PAHs). In this study, the automated cellular imaging system (ACIS) was used for localization and semi-quantitation of PAH-DNA adducts. Fresh tissue sections were cut from archived paraffin blocks and incubated with an antiserum elicited against DNA modified with 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene (BPDE). Nuclear PAH-DNA adduct staining was observed in four out of five human samples incubated with the anti-BPDE-DNA. By visual inspection, nuclei in the basal layer of the esophageal epithelium had higher levels of PAH-DNA adducts compared to those found in the adjacent superficial squamous layer. Nuclear PAH-DNA staining was absent in serial sections incubated with either normal rabbit serum or BPDE-DNA-antiserum previously absorbed with the immunogen BPDE-DNA. Semi-quantitative evaluation by ACIS revealed that per nucleus values for PAH-DNA adducts in the basal layer of the esophageal epithelium were 5- to 40-fold higher than those in the adjacent superficial squamous layer ($P < 0.0001$, using a random effects model). This pilot study demonstrates the presence of PAH-DNA adducts in archived paraffin-embedded endoscopic esophageal biopsy samples that are close to 20 years old, and suggests that an appropriate set of archived samples could be used to prospectively correlate PAH-DNA adduct formation with risk of esophageal cancer development.

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Abbreviations: ACIS, automated cellular imaging system; AU, arbitrary unit of staining intensity; BP, benzo[*a*]pyrene; BPDE, 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline

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1. Introduction

In Linxian, China, the cumulative mortality rate (~20%) from esophageal and proximal stomach cancer is possibly the highest seen anywhere in the world [1]. Historical records indicate that an elevated esophageal cancer rate has been present in this region for at least 2000 years [1]. For approximately 20 years, epidemiologists have examined hypotheses relating to potential etiologic factors in Linxian that include nutritional deficiencies, poor oral hygiene, exposure to nitrosamines and microbial contamination [1–4]. However, a single predominant etiologic factor has yet to be identified. The fact that Linxian residents have, for generations, heated their dwellings and cooked food on an open hearth using coal and wood, has lead to the hypothesis that elevated levels of ambient carcinogenic polycyclic aromatic hydrocarbons (PAHs) may contribute to the cancer risk observed in this region. This notion is further supported by identification of high levels of benzo[a]pyrene, a PAH and a known human carcinogen, in raw and cooked food obtained in Linxian [5]. Furthermore, high concentrations of the PAH urinary metabolite 1-hydroxypyrene were found in urine from Linxian residents [6], and PAH-DNA adducts were visualized by semi-quantitative immunohistochemistry in esophageal resection specimens obtained in Linxian in 1995 [7].

To explore a potential association between esophageal PAH-DNA adduct formation and esophageal cancer risk, one must be able to evaluate PAH-DNA adduct formation in samples of human esophagus taken one to three decades prior to cancer diagnosis. In this study, we used semiquantitative immunohistochemistry to examine archived esophageal biopsy material, taken from Linxian residents in 1985, for the presence of PAH-DNA adduct formation. The samples were part of an endoscopic survey in a large prospective epidemiologic study of cancer risk that includes careful patient follow-up and documentation of cancer development since 1985. The current pilot study was designed to examine archived esophageal endoscopic biopsy samples from Linxian for the presence of PAH-DNA adducts. Robust staining for PAH-DNA adducts was found in most of the samples taken in 1985, confirming the long-term persistence of PAH-DNA adducts in human paraffin-embedded

tissue and the feasibility of a larger prospective nested case-control study.

2. Materials and methods

2.1. Chemicals, reagents and antisera

Antigen retrieval citra solution, normal rabbit serum and the microwave pressure cooker were purchased from BioGenex, (San Ramon, CA). Permunt mounting media was from Fisher (Pittsburgh, PA). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody was from Dako (Glostrup, Denmark). The Ventana enhanced alkaline phosphate red paraffin staining kit and the hematoxylin counterstaining kit were purchased from Ventana (Tucson, AZ).

Antiserum specific for DNA modified with 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene (BPDE) (rabbit #31 bleed 8/16/78) was elicited and characterized as described [8–10]. The antiserum has specificity for the different enantiomers of benzo[a]pyrene (BP) bound to DNA, but no specificity for BP alone, BP metabolites, BP bound to protein or DNA alone [8]. Cross-reactivity of this antiserum for DNA samples modified with chrysene and other carcinogenic PAHs has been reported [10]; therefore, when using human samples that are likely to contain adducts of multiple PAHs, the values obtained are designated PAH-DNA adducts.

To prepare immunogen-absorbed BPDE-DNA antiserum (i.e. antiserum that contains no anti-BPDE-DNA), 0.4 ml of BPDE-DNA antiserum was absorbed two times with a total of 388 μ g BPDE-DNA (1.4% modified, 13.9 nmol BPDE-DNA adducts). After each incubation, the antigen-antibody complex was removed by centrifugation, and the final supernatant was used for staining.

2.2. Patients, tissue samples and primate controls

The five human esophageal samples from China were obtained from biopsies performed in Linxian in 1985. Tissues were fixed in 10% formalin and embedded in paraffin. The study was approved by the Institutional Review Boards of the collaborating institutions: The Cancer Institute of the Chinese Academy of Medical Sciences, Beijing, China, and

the National Cancer Institute, National Institutes of Health, Bethesda, MD.

2.3. Immunohistochemistry

For immunohistochemistry, each paraffin block was cut so that four tissue slices were positioned on each slide, and several slides were prepared from each sample. For each experiment, parallel slides were stained with: BPDE-DNA antiserum; BPDE-DNA-absorbed BPDE-DNA antiserum from which virtually all of the specific activity had been removed; or, normal rabbit serum.

Staining for PAH-DNA adducts in human esophagus was performed using the Nexes IHC (Ventana, Tucson, AZ) automated slide staining system. Before staining, fresh-cut sections (5 µm) of paraffin embedded esophagus were deparaffinized, hydrated and washed as previously described [7]. Subsequently, slides were microwaved in antigen retrieval citra solution (Biogenex) in a microwave pressure cooker (Biogenex) according to the manufacturer's directions. After the pressure was released and the pressure cooker was opened, slides were cooled for 20 min, rinsed in deionized water and loaded into the Nexes IHC automated slide stainer (Ventana). Staining was accomplished using the enhanced alkaline phosphate red paraffin staining kit (Ventana) and the hematoxylin counterstaining kit (Ventana). The system was set to incubate the primary antibody, which could be anti-BPDE-DNA (1:1000), BPDE-DNA-absorbed BPDE-DNA antiserum (1:1000) or normal rabbit serum (1:1000), for 20 min. Stained slides were rinsed in water containing Dawn soap for 1 min, and subsequently rinsed in tap water to remove the soap. Stained slides were air dried and mounted with Permount mounting media (Fisher) before the initial evaluation using a Nikon Eclipse (E400) microscope.

2.4. Semi-quantitation of nuclear staining intensity by automated cellular imaging system (ACIS)

The intensity of the nuclear PAH-DNA adduct staining (pink color) was measured using the ACIS, for which methodological details have been previously described [7]. This system consists of computer-directed components that include a bright field microscope, a charge coupled display digital camera, and an auto-

mated slide loading system. The ACIS software (version 1.81b and 1.82) regulates microscope calibration and slide loading, and runs applications that identify and quantify nuclear immunohistochemical staining.

Characterization of the immunohistochemical staining is based on three color parameters: the color defined by *hue* (pink is 190–224, blue is 130–190), the 'darkness' defined as *luminosity*, and density of the color defined as the *saturation*. The three parameters together comprise color intensity, with values expressed as arbitrary units (AUs). By increasing or decreasing the thresholds for *hue*, *luminosity* and *saturation*, it is possible to eliminate the contribution of background staining. For these experiments, the computer was programmed to determine intensity (AU) only in round or oblong shaped organelles within size range for nuclei found in esophageal epithelium.

For the quantitation of PAH-DNA adduct staining, the 'histological reconstruction' feature of the ACIS software was used. Initially, the section was visualized as a whole. Subsequently, the section was arbitrarily (but consistently) divided into two layers: the basal layer, that is the area containing crowded, large round nuclei located above the basement membrane; and the superficial squamous layer, that is the area containing sparse, flattened nuclei, including all the nuclei not designated as "basal". Each of the four tissue sections was divided into six to eight regions: three to four basal regions and three to four superficial squamous regions. Each region was analyzed, and the resulting values were combined and averaged to produce intensity values for basal and superficial squamous areas separately, as well as for the whole histologic section. In each section about 4000–8000 nuclei were measured. In slides stained with anti-BPDE-DNA or immunogen-absorbed anti-BPDE-DNA, the ACIS software determined the total number of pink nuclear pixels, indicating PAH-DNA adducts, and separately calculated the intensity of the pink color. In a set of parallel slides stained with normal rabbit serum and hematoxylin, the ACIS software determined the total number of blue nuclear pixels, indicating the total number of nuclei. These values were used to calculate the average pink staining intensity per nucleus and the percentage of nuclei that are positive for PAH-DNA adducts.

Designation of basal and superficial squamous layers was based on nuclear shape as an indicator of

proliferation status. In preliminary experiments, sections were stained with antiserum specific for PCNA [11] and the rounded cells in the basal layer were proliferating, while the flattened cells in the suprabasal squamous layer were not proliferating. Areas for the histological reconstruction were designated *basal* and *squamous* based on these criteria.

2.5. Statistical methods

We compared BPDE-DNA staining measured by ACIS for superficial squamous and basal areas of the esophageal epithelium using a random effects model [12]. On log-transformed ($\log [x + 1]$) data. The random effects model included cell-type as a fixed effect and a random intercept to account for between sample variation. All p values were two-sided and statistical tests were performed using likelihood-ratio tests.

3. Results

3.1. PAH-DNA adduct staining in human endoscopic esophageal samples from Linxian

Esophageal endoscopic biopsy samples from five inhabitants of Linxian were procured in 1985 and stored embedded in paraffin. Fresh 5 μm sections from each block were cut, incubated with normal rabbit serum (Fig. 1A and D) or immunogen-absorbed BPDE-DNA antiserum (Fig. 1B and E), and counter-stained with hematoxylin; these slides showed no pink nuclear staining, indicating an absence of PAH-DNA adducts. However when slides were stained with the BPDE-DNA antiserum, intense pink nuclear staining indicating the presence of PAH-DNA adducts (Fig. 1C

and F) was observed in sections from four of the five individuals. Because the BPDE-DNA antiserum has cross-reactivity for DNA samples modified with several PAHs [10], a positive signal in human samples is considered to indicate the formation of not only BPDE-DNA adducts but also DNA adducts of other PAHs. In these experiments, the staining in Fig. 1C and F is considered specific for PAH-DNA adducts because pre-absorption of the serum with the original immunogen, BPDE-DNA, removed essentially all of the pink staining (Fig. 1B and E).

3.2. PAH-DNA semi-quantitation by ACIS

Four sequentially-cut esophageal tissue sections from the same paraffin block were placed on a single microscope slide and after staining each section was quantified separately by ACIS. Fig. 2 shows the combined mean AU/1000 nuclei for whole tissue sections ($n = 4$) obtained from each of five individuals and stained with either BPDE-DNA antiserum (\square) or immunogen-absorbed BPDE-DNA antiserum (\blacksquare). For the BPDE-DNA antiserum staining, one sample (349A) had a very low PAH-DNA value (3 AU/1000 nuclei), while the others varied between 12 and 54 AU/1000 nuclei, a difference of less than five-fold. Values for the sections stained with immunogen-absorbed serum ranged between 0 and 2 AU/1000 nuclei, indicating the specificity of the BPDE-DNA antiserum for PAH-DNA adducts.

In the Tables, ACIS values for the esophageal epithelium basal layer (Table 1) and superficial squamous layer (Table 2) for each of the five study subjects are shown separately. PAH-DNA values in the basal layer were ~ 4 - to 40-fold higher than PAH-DNA values in the superficial squamous layer for the same tis-

Table 1
PAH-DNA staining in the basal layer of human esophagus from Linxian obtained in 1985

Sample number	Number of cells examined	BPDE-DNA serum ^a (AU/1000 nuclei mean \pm S.E., $n = 4$)	Absorbed serum ^b (AU/1000 nuclei, mean \pm S.E., $n = 4$)
151A	1376	87.79 \pm 7.56	0.29 \pm 0.22
249N	1909	22.16 \pm 2.30	4.03 \pm 2.67
349A	1692	5.80 \pm 0.77	0.00 \pm 0.00
436A	1822	24.20 \pm 2.03	0.00 \pm 0.00
52A	1787	42.42 \pm 1.40	0.28 \pm 0.11

^a Differences between basal cell and superficial squamous cell PAH-DNA AU values, $P < 0.0001$, using a random effects model with a likelihood-ratio test (chi-square = 36, d.f. = 1).

^b BPDE-DNA antiserum absorbed with immunogen BPDE-DNA.

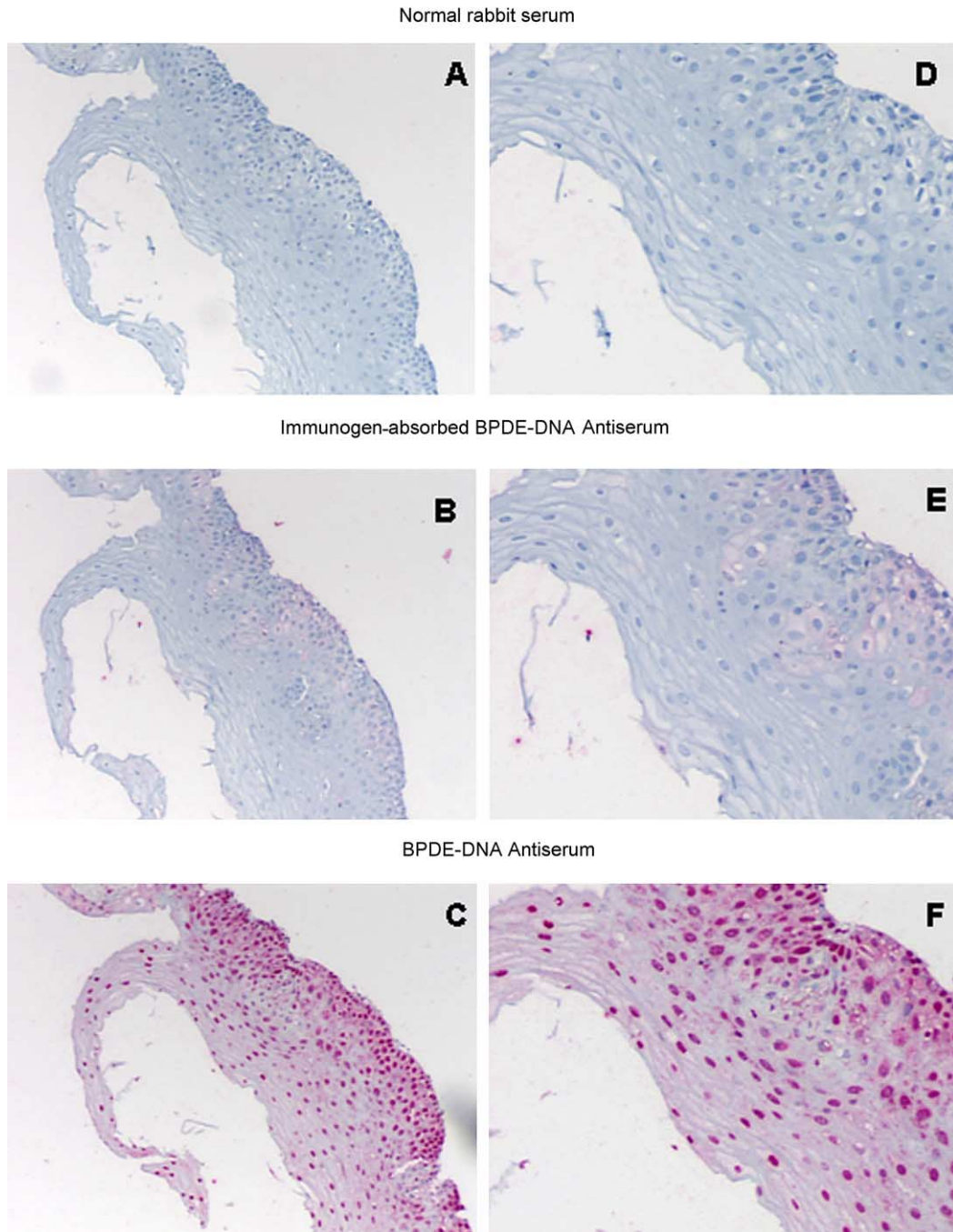


Fig. 1. Immunohistochemical staining for human esophageal epithelium (sample 151A) obtained in Linxian in 1985. Photomicrographs at 250 \times magnification (A–C) and 500 \times magnification (D–F); A and D are stained with normal rabbit serum; B and E are stained with immunogen-absorbed anti-BPDE-DNA containing no specific BPDE-DNA antiserum; C and F are stained with anti-BPDE-DNA. For nuclei in A, B, D and E blue color represents hematoxylin staining; in C and F nuclear pink color represents PAH-DNA adduct staining with fast red.

Table 2

PAH-DNA staining in the superficial squamous layer of human esophagus from Linxian obtained in 1985

Sample number	Number of cells examined	BPDE-DNA serum ^a (AU/1000 nuclei mean \pm S.E., $n = 4$)	Absorbed serum ^b (AU/1000 nuclei, mean \pm S.E., $n = 4$)
151A	1931	20.14 \pm 4.50	0.00 \pm 0.00
249N	3625	1.32 \pm 0.19	0.03 \pm 0.03
349A	4551	0.51 \pm 0.02	0.00 \pm 0.00
436A	3272	3.21 \pm 0.49	0.06 \pm 0.03
52A	6307	1.11 \pm 0.13	0.08 \pm 0.03

^a Differences between basal cell and superficial squamous cell PAH-DNA AU values, $P < 0.0001$, using a random effects model with a likelihood-ratio test (chi-square = 36, d.f. = 1).

^b BPDE-DNA antiserum absorbed with immunogen BPDE-DNA.

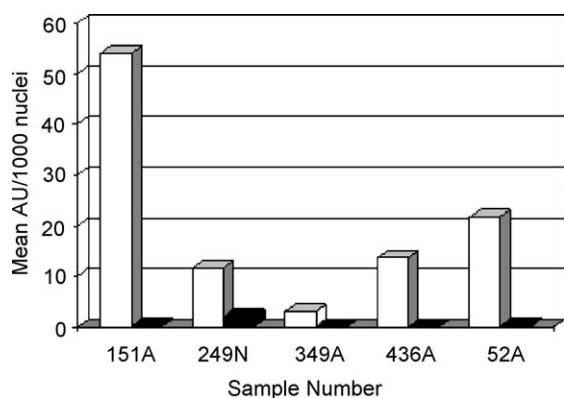


Fig. 2. Semiquantitative determination (ACIS) of PAH-DNA adducts in esophageal epithelium from Linxian. Bars are means for four tissue samples stained with either anti-BPDE-DNA (\square) or immunogen-absorbed anti-BPDE-DNA (\blacksquare). Values for anti-BPDE-DNA staining (AU/1000 cells \pm S.E., $n = 4$) are 54 ± 6 , 12 ± 1 , 3 ± 0.4 , 14 ± 1 and 22 ± 2 for samples 151A, 249N, 349A, 436A and 52A, respectively. Mean values for immunogen-absorbed anti-BPDE-DNA staining (AU/1000 cells, $n = 4$) are 0.1, 2.0, 0, 0 and 0.2 for samples 151A, 249N, 349A, 436A and 52A, respectively.

sue section. Tables 1 and 2 also show that incubation of parallel sections with BPDE-DNA antiserum absorbed with the immunogen BPDE-DNA reduces the staining to essentially background levels, indicating that the positive staining was specific for PAH-DNA adducts.

4. Discussion

In human esophagus from Linxian, China, a high-risk region for esophageal carcinoma, nuclear staining

for PAH-DNA adducts was detectable in freshly-cut sections from paraffin-embedded endoscopic biopsy blocks obtained in 1985. The nuclear staining was strong, and quantitation by the ACIS imaging system showed good reproducibility for the four tissue sections stained simultaneously on a single slide. In addition, nuclear staining was absent when parallel sections were incubated with normal rabbit serum and immunogen-absorbed BPDE-DNA antiserum, confirming the specificity of PAH-DNA adduct staining.

In the Linxian samples, higher PAH-DNA levels were found in the basal layer of the esophageal mucosa, compared to the superficial squamous layers. This finding is consistent with the known biology of this tissue, as cells in the basal layer are growing and proliferating while cells in the squamous layer are undergoing maturation and senescence [14]. Our observation that cells in the replicating basal layer had more PAH-DNA adducts than those in the superficial quiescent squamous layer cannot be explained by the data shown here, but are not likely to be due to differential rates of adduct degradation during storage of the paraffin blocks. One could speculate that the difference may be partially explained by cell proliferation; that is, the open configuration of replicating DNA in the basal layer would allow the DNA bases to be readily accessible for electrophilic carcinogen adduction [15]. In addition, studies with confluent, quiescent normal human fibroblasts have shown that DNA adducts of benzo[a]pyrene continue to be removed by nucleotide excision repair [16], suggesting that cells in the superficial squamous layers may have the capacity to remove PAH-DNA adducts during senescence. In the esophageal squamous epithelium, similar to the skin, tumors are likely to be clonal expansions of mutated

basal cells within the most highly-proliferative compartment [13].

Previously van Gijssel et al. [7] validated the ACIS for semi-quantitation of PAH-DNA adduct immunohistochemical staining. A standard curve was prepared from paraffin-embedded sections of cultured human keratinocytes exposed to increasing concentrations of BPDE, and values for nuclear staining intensity correlated directly with BPDE exposure concentrations. In addition, the BPDE-DNA chemiluminescence immunoassay [9] was used to assay DNA extracted from similarly-exposed cultures, and the observed BPDE-DNA adduct levels correlated both with BPDE exposure concentration and AU values determined by ACIS. Among other things, the cell culture studies demonstrated that ACIS evaluation is linear over a broad range of both BPDE and BPDE-DNA adduct concentrations.

A central question in the epidemiology of human cancers caused by chemicals is the association between DNA adduct formation and subsequent cancer risk. A meta-analysis of several case-control studies has shown that current smokers with high levels of bulky DNA adducts, determined by ^{32}P postlabeling, were at increased risk of lung and bladder cancers [17]. Among current smokers, the cases had 87% higher bulky DNA adducts, compared to the controls, with statistically significant associations for both the meta-analysis and five of seven individual studies. A potentially more robust epidemiological framework, the prospective nested case-control study design, has rarely been used to examine the association between DNA adducts and cancer risk, because of the difficulty of obtaining appropriately archived material. There are, however, successful studies of this type for smoking and lung cancer, as well as for aflatoxin exposure and liver cancer. Utilizing 15,700 blood samples collected for the Physicians' Health Study, Tang et al. [18] showed that among current smokers (cases = 36, controls = 64), lung cancer risk was significantly associated with bulky DNA adducts determined by ^{32}P postlabeling for adducts stratified as high versus low (odds ratio, OR = 2.98) and for adduct values treated as a continuous variable (OR = 4.65 for 2 logs unit increase). Similarly, a study of excreted aflatoxin-B₁-N⁷-guanine and risk of hepatocellular carcinoma by Qian et al. [19] using urine samples archived from 18,244 men

in Shanghai, China [19,20] individuals excreting aflatoxin-B₁-N⁷-guanine in the urine had a 9.1-fold increased risk for liver cancer compared to unexposed individuals. In the above studies, target organ tissues were not available prior to cancer diagnosis, and the use of surrogate tissues was mandatory. In this pilot study, the feasibility of determining PAH-DNA adducts in endoscopically obtained esophageal biopsies taken about 20 years ago has been demonstrated, suggesting the possibility that similar archived samples could be used to prospectively correlate PAH-DNA adduct formation with risk of subsequent esophageal cancer development. In addition, the study showed that, among exposed individuals, PAH-DNA adducts appear to be concentrated in the basal layers of the superficial squamous mucosa. The prognostic significance of this localization warrants further exploration.

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